AMITY UNIVERSITY
RAJASTHAN

AMITY INSTITUTE OF MICROBIAL TECHNOLOGY

Dissertation Submitted in the Partial Fulfilment of the Requirement for

M.Sc. (Industrial Microbiology)

Semester – IV

2024-2026

“Real-Time Monitoring of Yeasts During Fermentation Using HPLC-RI”

Submitted By: Himanshu **Submitted To: Dr G.K Aseri**

Name of Student -Himanshu Mishra, Sanjeev Kumar
Enrolment. No. -A20229224002

Name of Supervisor – Dr. Pooja
Designation – Senior Scientist

Format as follows: -

A. Cover page (Outside & inside)

B. Certificate from Industry/research place

C. Acknowledgement

D. Index (In tabulate form)

E. List of Figures

F. List of Tables

G. Abbreviations

H. Summary

1.Introduction

2.Review of Literature

3.Methodology

4.Result and Discussion

5.Conclusion

6. References

7. Annexure (if any)

Sample Index

S. No.	Title	Page No.
1	Introduction	1
1.1	Introduction to Chromatography	1
1.2	Classification of Chromatographic Techniques	2
1.3	High-Performance Liquid Chromatography (HPLC)	3
1.4	HPLC Instrumentation and Working Principle	3
1.5	Detectors Used in HPLC	3
1.6	Importance of RID in Fermentation Analysis	4
1.7	Fermentation and Yeast Metabolism	4
1.8	Changes During Fermentation Process	5
1.9	Need for Real-Time Monitoring in Fermentation	5
1.10	Application of HPLC-RID in Fermentation Industry	6
1.11	Scope and Objective of Study	6
2	Review of Literature	7
2.1	Yeast Fermentation and Sugar Metabolism	7
2.2	Fermentation Kinetics and Process Monitoring	7
2.3	Analytical Techniques for Fermentation Monitoring	7
2.4	Application of HPLC in Fermentation Analysis	8
2.5	Role of Refractive Index Detector in Sugar Analysis	8
2.6	HPLC-RI in Fermentation Monitoring	9
2.7	Real-Time Monitoring of Fermentation Processes	9
2.8	Research Gap	10
3	Methodology	11
3.1	Study Area	11
3.2	Chemicals and Reagents	12
3.3	Instruments and Equipment	13
3.4	Sample Description	17
3.5	Preparation of Standard Solutions	19
3.6	Analytes Studied in Fermentation Samples	20
3.7	Sample Preparation	21
3.7.1	Preparation of Molasses Samples	21
3.7.2	Preparation of Grain-Based Fermentation Samples	22
3.7.3	Filtration and Sample Clarity	23
3.7.4	HPLC Injection	24
3.8	Chromatographic Conditions	25

4	Results and Discussion	26
5	Conclusion	48
6	References	50

Summary

The preparation and chromatographic analysis of fermentation samples based on grain and molasses using High Performance Liquid Chromatography in conjunction with a Refractive Index Detector (HPLC-RID) was the main focus of this investigation. Before chromatographic analysis, fermentation samples were diluted, vortexed, centrifuged, and filtered to reduce matrix interference and increase analytical precision. Under ideal chromatographic conditions, sulfuric acid was used as the mobile phase for the analysis of the produced samples. Due to variations in substrate composition and fermentation activity, distinct chromatographic patterns were seen for samples of grain and molasses fermentation. Throughout the fermentation process, the amounts of ethanol and leftover carbohydrate components were successfully tracked. The chromatograms confirmed the applicability of HPLC-RID for routine monitoring of industrial fermentation samples by showing efficient separation and trustworthy detection of fermentation-related components. The study emphasizes how crucial proper sample preparation is to achieve consistent chromatographic performance and repeatable analytical outcomes.

Keywords

High-Performance Liquid Chromatography (HPLC), Fermentation, Sugar Utilization, Ethanol Production, Fermentation Metabolites

CHAPTER 1: Introduction

1.1 Introduction to Chromatography

Chromatography is an analytical method in chemistry that enables the identification, separation, and quantification of compounds in complex mixtures. It is a high sensitivity and specificity technique commonly used in pharmaceutical, food, environmental, biochemical, and industrial investigations. Chromatography has a basic principle of how compounds interact with the stationary and mobile phases. Based on distinct chemical properties, substances travel at varying rates, resulting in their separations (Skoog et al., 2018).

The development of chromatographic techniques that allow fast and precise analysis of sugars, alcohols, proteins, amino acids, and organic acids has significantly improved analytical science. Chromatographic methods have been developed over the years for different analytical purposes. These include paper chromatography, thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC). HPLC is among these methods and has become one of the most reliable and popular techniques because of its high resolution, reproducibility, and ability to analyse thermally unstable and non-volatile compounds (Dong, 2006).

Chromatographic methods are especially important in fermentation industries where there is a need for continuous monitoring of substrate and products. During fermentation, microorganisms convert sugars into products such as ethanol, glycerol, and organic acids. Such changes need to be checked in order to understand fermentation kinetics, perfect process conditions, and improve product quality. Analytical techniques such as HPLC provide correct data on the concentration of sugars and metabolites at various stages of fermentation.

1.2 Classification of Chromatographic Techniques

Chromatographic methods can be classified according to the nature of the stationary phase, mobile phase, and separation mechanism. The common chromatographic methods are adsorption chromatography, partition chromatography, ion-exchange chromatography, size-exclusion chromatography, gas chromatography, and liquid chromatography. Paper chromatography and TLC are simple, inexpensive methods used for preliminary analysis. Gas chromatography provides excellent separation of volatile compounds but requires elevated temperatures and extensive sample preparation.

By contrast, liquid chromatography is useful for the analysis of compounds that are non-volatile and thermally unstable and is especially useful in biological and fermentation studies (Snyder et al., 2012).

High-performance liquid chromatography (HPLC) is a more sophisticated form of liquid chromatography that uses high pressure to force solvents through tightly packed columns. The technique has better separation efficiency, less analysis time, and better sensitivity than conventional liquid chromatography. The above advantages make HPLC widely used in industrial fermentation monitoring, food quality analysis, pharmaceutical research, and biochemical studies.

1.3 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography is one of the most powerful analytical techniques for qualitative and quantitative analysis of compounds present in liquid samples. HPLC is based on the differential interaction of compounds between the stationary phase and the mobile phase. As the sample traverses the column, compounds are separated by polarity, molecular size, and affinity to the stationary phase (Meyer, 2010).

The basic components of an HPLC system are solvent reservoir, pump, injector, chromatographic column, detector, and data acquisition system. The mobile phase under high pressure forces the sample through the column, and separation occurs. The separated compounds are then

detected and recorded as peaks on a chromatogram. The technique is highly preferred due to the following:

- High sensitivity
- Excellent reproducibility
- Rapid analysis
- Accurate quantification

The ability to analyse multiple compounds simultaneously with HPLC has become extremely critical in fermentation analysis because fermentation broths have complex mixtures of sugars, alcohols, and organic acids, which need to be separated and quantified efficiently.

1.4 HPLC Instrumentation and Working Principle

The proper operation of HPLC's main components is essential to its performance. Throughout the analysis, a steady flow of mobile phase is kept via the solvent delivery system or pump. While the chromatographic column separates substances according to how they interact with the stationary phase, the injector adds a predetermined volume of material to the system.

As separated substances elute from the column, the detector recognizes them and transforms the signal into chromatographic peaks. Computer software is used to examine the produced chromatograms in order to find and quantify the chemicals.

In this work, fermentation samples were checked in real time using a Shimadzu HPLC system in conjunction with a refractive index detector (RID). Under carefully regulated working conditions, the 0.005 M sulfuric acid mobile phase effectively separated sugars, ethanol, and organic acids.

1.5 Detectors Used in HPLC

Depending on the type of analytes, several detectors are employed in HPLC. Ultraviolet (UV), photodiode array (PDA), fluorescence, conductivity, and refractive index detectors are examples of common detectors.

Due to their simplicity and great sensitivity, UV detectors are widely used. However, UV detection is less useful for such investigations since several fermentation products, including sugars and ethanol, show low UV absorbance. Because they can find substances without UV absorption, refractive index detectors are consorted for the study of alcohol and carbohydrates (Dong, 2006).

RID measures variations in the mobile phase's refractive index as analytes flow through the detector cell. When steady operating conditions are kept, RID offers accurately of sugars, ethanol, glycerol, and organic acids despite its sensitivity to temperature and pressure changes.

1.6 Importance of RID in Fermentation Analysis

Refractive index detection is widely used in fermentation studies because it allows simultaneous analysis of sugars and fermentation products in a single chromatographic run. Fermentation samples often have glucose, fructose, sucrose, ethanol, glycerol, lactic acid, and acetic acid, all of which can be effectively detected using RID.

The technique provides several advantages, including:

- Simple operation
- Good reproducibility
- Cost-effectiveness
- Simultaneous detection of multiple compounds
- Minimal sample preparation

In fermentation industries, RID-based HPLC methods are commonly applied for monitoring sugar use and ethanol formation during alcoholic

fermentation. Continuous monitoring helps in evaluating yeast activity, fermentation efficiency, and substrate conversion.

1.7 Fermentation and Yeast Metabolism

Under carefully regulated environmental conditions, microbes transform organic substrates into simpler molecules through the biochemical process of fermentation. Because yeast can effectively convert sugars into carbon dioxide and ethanol, it plays a significant role in industrial fermentation.

The most used yeast species in fermentation industries is Saccharomyces cerevisiae because of its quick growth rate, strong ethanol tolerance, and effective sugar metabolism (Walker, 2011). Through the process of glycolysis, yeast uses fermentable carbohydrates to produce pyruvate as an intermediate product during fermentation. Pyruvate is transformed into carbon dioxide and ethanol in anaerobic environments.

Secondary metabolites like glycerol, lactic acid, acetic acid, and higher alcohols are produced by yeast in addition to ethanol. These metabolites affect the efficiency of fermentation and the properties of the finished product. Yeast's metabolic activity is affected by factors such as temperature, pH, nutrient concentration, oxygen availability, and substrate composition.

1.8 Changes During Fermentation Process

The dynamic process of fermentation involves ongoing metabolic and biochemical alterations. In the lag phase, yeast first adjusts to the fermentation environment. The exponential phase, which is characterized by fast sugar consumption and ethanol generation, comes next.

As fermentation proceeds, ethanol and other metabolites rise as sugar concentration falls. Comprehend fermentation kinetics and assess process performance, it is critical to keep an eye on these changes. Incomplete sugar use, a lower ethanol yield, and the production of undesirable byproducts can all result from improper fermentation conditions. For industrial fermentation operations to be perfected, these characteristics are crucial (Boulton & Quain, 2006).

Monitoring fermentation in real time enables improved comprehension of:

Patterns of sugar intake

Production trends for ethanol

creation of organic acids

Activity of yeast metabolism

efficiency of fermentation

These parameters are important for optimizing industrial fermentation processes.

1.9 Need for Real-Time Monitoring in Fermentation

Continuous or sporadic study of fermentation parameters throughout the process is referred to as real-time monitoring. Maintaining process stability, product quality, and fermentation efficiency all depend on correct monitoring.

Conventional analytical methods like titration and spectrophotometry may not be extremely specific and offer little information. With little sample preparation, HPLC-RID offers extremely precise and repeatable analysis of fermentation metabolites.

The use of HPLC-RID in fermentation monitoring helps in:

- Detecting sugar depletion
- Measuring ethanol production
- Monitoring organic acid formation
- Evaluating fermentation kinetics
- Finding process deviations

Continuous monitoring also helps industries improve productivity and reduce economic losses caused by incomplete fermentation or contamination.

1.10 Application of HPLC-RID in Fermentation Industry

HPLC-RID is widely used in the food processing, biotechnology, brewing, and bioethanol production industries. The method is often used to measure the amounts of lactic acid, acetic acid, ethanol, glycerol, glucose, fructose, and sucrose in fermentation broths.

HPLC-RID is a great tool for fermentation research since it can evaluate several molecules at once. Chromatographic analysis offers crucial information on product formation, substrate usage, and fermentation performance in industrial applications.

In order to assess changes in sugars and fermentation products during various phases of fermentation, the current study employed HPLC-RID for real-time monitoring of fermentation samples based on grain and molasses.

1.11 Scope and Objective of the Study

The present study focuses on the real-time monitoring of yeast during fermentation using HPLC-RID. The study aims to analyse the changes in sugars and fermentation metabolites during the fermentation process.

The major aims of the study include:

- Preparation and analysis of fermentation samples
- Monitoring sugar use during fermentation
- Detection of ethanol and organic acids using HPLC-RID
- Evaluation of fermentation behaviour in grain and molasses samples

CHAPTER 2: Review of Literature

2.1 Yeast Fermentation and Sugar Metabolism

Yeast-mediated fermentation is a central biochemical process in which sugars are metabolized into ethanol and carbon dioxide through glycolysis and later alcoholic fermentation pathways. *Saccharomyces cerevisiae* stays the most widely used organism due to its robustness and high ethanol tolerance. According to Graham M. Walker (2011), yeast efficiently uses hexose sugars such as glucose and fructose, converting them into pyruvate under anaerobic conditions.

Recent studies have further highlighted the role of sugar transport systems and enzymatic regulation in fermentation efficiency. For example, research by Isak S. Pretorius et al. (2019) proved that the rate of sugar uptake significantly influences ethanol yield and by-product formation. The study emphasized that efficient sugar metabolism is linked to fermentation performance.

2.2 Fermentation Kinetics and Process Monitoring

Fermentation kinetics describes the rate of substrate consumption and product formation during the fermentation process. These kinetics are influenced by multiple factors, including temperature, pH, nutrient availability, and yeast strain.

According to Jens Nielsen et al. (2017), monitoring fermentation kinetics is essential for perfecting industrial processes and ensuring consistent product quality. The authors highlighted that real-time data on glucose

depletion and ethanol production allows better control of fermentation systems.

More recent work by Zhang et al. (2020) showed that continuous monitoring of fermentation parameters can significantly improve process efficiency by enabling early detection of deviations and optimizing operational conditions.

2.3 Analytical Techniques for Fermentation Monitoring

Several analytical methods have been employed for monitoring fermentation processes, each with specific advantages and limitations. Spectrophotometric methods are simple and rapid but lack specificity, especially when multiple compounds are present.

Gas chromatography (GC) provides high sensitivity and resolution but requires derivatization steps for non-volatile compounds, making it less suitable for sugar analysis. According to Michael W. Dong (2006), high-performance liquid chromatography (HPLC) offers a more versatile approach due to its ability to analyse a wide range of compounds without extensive sample preparation.

Recent advancements have also introduced biosensors and online monitoring systems; however, HPLC is a gold standard for correct quantification of fermentation metabolites (Silva et al., 2021).

2.4 Application of HPLC in Fermentation Analysis

HPLC has been widely applied in fermentation studies for the separation and quantification of sugars, alcohols, and organic acids. Its high resolution and reproducibility make it suitable for complex fermentation matrices.

A study by Moldoveanu and David (2019) proved the effectiveness of HPLC in analysing multiple fermentation components simultaneously, including glucose, fructose, and ethanol. The authors reported that HPLC provides precise and reliable results, making it an essential tool in fermentation research.

Additionally, recent studies have utilized HPLC for checking dynamic changes in fermentation systems, enabling better understanding of microbial activity and process kinetics (Kumar et al., 2022).

2.5 Role of Refractive Index (RI) Detector in Sugar Analysis

The refractive index detector is widely used in HPLC for detecting compounds that lack ultraviolet absorbance, such as sugars and alcohols. Unlike UV detectors, RI detectors measure changes in the refractive index of the eluent as analytes pass through the detector.

According to Dong (2006), RI detection is particularly suitable for carbohydrate analysis due to its universal response to solutes. However, the technique is sensitive to temperature and pressure variations, which can affect baseline stability.

Recent improvements in detector design have enhanced the stability and sensitivity of RI detectors, making them more dependable for routine fermentation analysis (Patel et al., 2021).

2.6 HPLC-RI in Fermentation Monitoring

HPLC-RI has gained significant importance in fermentation studies due to its ability to simultaneously quantify sugars and fermentation products. It is commonly used to check glucose, fructose, sucrose, and ethanol during fermentation.

A study published in *Food Chemistry* (2019) proved that HPLC-RI provides accurate measurements of sugar concentrations in fermented products. The authors concluded that the method is highly suitable for tracking fermentation progress.

Similarly, research by Singh et al. (2022) showed that HPLC-RI can be effectively used for monitoring fermentation kinetics in yeast-based systems. The study highlighted its application in evaluating sugar depletion and ethanol formation over time.

2.7 Real-Time Monitoring of Fermentation Processes

Real-time monitoring of fermentation processes has become increasingly important in modern biotechnology. It enables continuous tracking of fermentation parameters, allowing for improved process control and optimization.

According to Nielsen et al. (2017), real-time monitoring enhances process efficiency by providing immediate feedback on substrate consumption and product formation. Integration of analytical tools such as HPLC-RI into fermentation systems allows for correct and prompt data acquisition.

Recent advancements in automation and data analysis have further improved the feasibility of real-time monitoring. Studies have shown that combining HPLC with automated sampling systems can significantly enhance process reliability and reduce variability (Garcia et al., 2023).

2.8 Research Gap

Even though HPLC-RI is widely used in fermentation analysis, there are still a number of obstacles to overcome. Instead of actual real-time monitoring, the majority of studies concentrate on offline or batch analysis. There are still issues that need to be resolved, including detector sensitivity, sampling frequency, and system integration.

Additionally, little study has been done on how to perfect HPLC-RI systems for ongoing yeast fermentation monitoring. Improved industrial applications, more efficiency, and greater process control can result from addressing these issues.

CHAPTER 3: Methodology

3.1 Study Area

The present study was carried out in the chromatography laboratory of Catalysts Biotechnologies Private Limited, equipped with advanced analytical instruments for chromatographic and fermentation analysis. The laboratory provides controlled environmental conditions required for handling fermentation samples, preparation of analytical reagents, and operation of High-Performance Liquid Chromatography (HPLC) systems.

The major objective of the work was to monitor fermentation behaviour in grain-based and molasses-based samples using HPLC coupled with a Refractive Index Detector (RID). The laboratory setup allowed accurate separation and quantification of sugars, alcohols, oligosaccharides, and organic acids produced during fermentation.

The study mainly focused on analysing changes in sugar concentration and formation of fermentation metabolites such as ethanol, glycerol, lactic acid, and acetic acid during different stages of fermentation. Special attention was given to maintaining analytical accuracy, reproducibility, and sample integrity throughout the experimental work.

All experimental procedures including sample dilution, centrifugation, filtration, preparation of standard solutions, and chromatographic analysis were performed according to standard laboratory operating procedures. Proper safety measures and handling practices were followed during reagent preparation and instrument operation to minimize contamination and analytical errors.

The chromatography facility included essential instruments such as Shimadzu HPLC systems, centrifuges, micropipettes, analytical balances, vortex mixers, filtration assemblies, and autosampler units, which collectively supported the successful execution of the study.

3.2 Chemicals and Reagents

All chemicals and reagents used in the present investigation were of analytical or HPLC grade to ensure high purity and accurate chromatographic analysis. HPLC-grade water prepared using a Milli-Q water purification system was used throughout the experimental work for preparation of mobile phase, dilution of samples, and preparation of standard solutions.

Dilute sulfuric acid (0.005 Mm H_2SO_4) was used as the mobile phase during chromatographic analysis because of its effectiveness in separating sugars, alcohols, and organic acids under ion-exclusion chromatographic conditions. The mobile phase was freshly prepared, filtered, and degassed prior to use in the HPLC system to avoid baseline disturbances and pressure fluctuations.

Analytical reference standards including DP1, DP2, DP3, DP4, fructose, glycerol, lactic acid, acetic acid, and ethanol were used for calibration and identification of analytes in fermentation samples. These standards were prepared in suitable concentrations using calibrated volumetric glassware and micropipettes to ensure accuracy and reproducibility.

Syringe membrane filters (0.2 µm), HPLC vials, Eppendorf tubes, and laboratory-grade glassware were used during sample preparation and filtration procedures. All reagents and prepared solutions were properly labelled and stored under suitable laboratory conditions to maintain stability and prevent contamination.

Proper handling and preparation of chemicals played an important role in obtaining stable chromatographic conditions, clear peak separation, and reliable analytical results during HPLC-RID analysis.

Figure 3.1: Milli-Q water purification system used for the preparation of HPLC-grade water.

All reagents were stored under proper conditions, and fresh solutions were prepared. Proper labelling and handling practices were followed to keep traceability and consistency throughout the experimental process.

3.3 Instruments and Equipment

A Shimadzu HPLC system with a refractive index detector (RID) was used to conduct chromatographic analysis. Fermentation samples may be accurately and consistently analysed thanks to the system's solvent delivery unit, autosampler, column oven, and detector.

Because it allows the detection of substances like sugars and alcohols that do not absorb ultraviolet light, the refractive index detector proved especially useful for this investigation. Maintain steady baseline conditions and reliable retention times, the system's parameters were meticulously regulated.

A Shimadzu HPLC system (1220 series) with a refractive index detector (RID) was used for chromatographic analysis. A solvent delivery device, autosampler, column oven, and detector made up the system, which allowed for precise and repeatable examination of fermentation samples.

Because it allows the detection of substances like sugars and alcohols that do not absorb ultraviolet light, the refractive index detector proved especially proper for our investigation. In order to preserve steady baseline conditions and regular retention times, the system parameters were meticulously regulated.

Figure 3.2: Shimadzu HPLC system equipped with RID used for chromatographic analysis.

Chromatographic analysis of fermentation samples was performed using a Shimadzu HPLC system equipped with a Refractive Index Detector (RID). The instrument was used for the separation and monitoring of sugars, ethanol, glycerol, and organic acids present in grain-based and molasses-based fermentation samples.

Figure 3.3: Centrifuge used for sample clarification

During sample preparation, a number of auxiliary instruments were used in addition to the HPLC system. Cut suspended particles and produce a clear supernatant—which is crucial for avoiding column blockage—a centrifuge was used. Ensure correct volume measurements, samples were transferred and diluted using a micropipette.

Following centrifugation, a clear supernatant was left above the compact pellet that had formed at the tube's bottom. The pellet was not disturbed throughout the cautious collection of the supernatant, which was then used for filtration, HPLC analysis, and any necessary dilution. These procedures guaranteed the elimination of particulate matter, enhanced analytical precision, and prevented obstruction of the chromatographic column.

Figure 3.3: Micropipette used for sample transfer

A calibrated micropipette was used to dilute the fermented grain wash samples for examination. First, a clean Eppendorf tube was filled with 1.0 mL of mobile phase. The diluted solution (about 1:11 dilution) was then made by adding 0.1 mL of the sample to the same tube. After that, the mixture was vortexed for 30 to 60 seconds to guarantee total homogenization and even analyte distribution in the solution. Throughout the process, proper pipetting technique was used to ensure precision and prevent contamination. The material was prepared for added processing, such as centrifugation and filtration before HPLC analysis, after vertexing.

In order to avoid column overloading, enhance peak resolution, and guarantee correct chromatographic analysis, this dilution step was crucial.

Figure 3.4: HPLC autosampler tray with prepared sample vials

All instruments were run according to standard laboratory procedures, and necessary precautions were taken to maintkeepracy and reproducibility.

3.4 Sample Description

Both grain-based and molasses-based fermentation substrates made up the samples examined in this investigation. Grain-based samples were from fermentation techniques that involve microbial fermentation after enzymatic hydrolysis of source materials rich in starch. Molasses samples, on the other hand, were obtained from byproducts of the sugar industry that had high sucrose concentrations, which made them proper fermentation substrates.

In industrial fermentation processes, these two kinds of samples are typical feedstocks. Monitoring the usage of sugars and the development of fermentation products over time was the main focus of the analysis. Comparative analysis is crucial since these substrates differ greatly in composition, which also affects their metabolite profiles and fermentation behaviour. Preserve integrity and avoid contamination, all samples were managed accepted laboratory practices. Prevent deterioration or microbiological contamination, samples were stored under suitable conditions and examined within a predetermined time.

Sample Type	Sample Description	Purpose
Grain-based sample	Fermented grain wash	Monitor sugar use
Molasses sample	Sucrose-rich fermentation substrate	Monitor sugar breakdown

Table 3.1: Description of Samples

Both grain-based and molasses-based samples were analysed to study the chromatographic behaviour of different fermentation substrates. Grain-based samples represented starch-derived fermentation material, while molasses samples rested for sugar-rich substrate suitable for monitoring sugar breakdown and metabolite formation.

Figure 3.5: Samples of molasses

3.5 Preparation of Standard Solutions

Provide precise HPLC system calibration, standard solutions for sugars, oligosaccharides (DP1–DP4), organic acids, glycerol, and ethanol were made using analytical-grade reference materials. Using an analytical balance, each solid standard (DP fractions, fructose, lactic acid, glycerol, and acetic acid) was precisely weighed (~0.2 g, with the exception of acetic acid, ~0.1 g) and put into a volumetric flask. The stock solution was then prepared by adding mobile phase to get the volume up to 50 ml.

Since ethanol is a liquid standard, 2 mL was measured volumetrically and diluted to 50 mL using the mobile phase. All solutions were appropriately mixed to ensure complete dissolution and homogeneity.

Working calibration standards were prepared from the stock solution at six different concentration levels (Level 1 to Level 6) using serial dilution with calibrated micropipettes. These concentration levels were selected to cover the expected analytical range of the samples and to ensure linearity of detector response.

Proper care was taken during preparation, including the use of clean glassware, correct pipetting techniques, and thorough mixing, to avoid any variation in concentration. The prepared standards were used for both retention time identification and quantitative analysis through calibration curves.

Standard	Weight (g) / Volume (mL)	Final Volume (mL)
DP4+/DP4	0.2003	50
DP3	0.2	50
DP2	0.1994	50

DP1	0.1996	50
Fructose	0.2001	50
Lactic acid	0.2008	50
Glycerol	0.2006	50
Acetic Acid	0.1008	50
Ethanol	2	50

Table 3.2: Preparation of Standard Stock Solutions

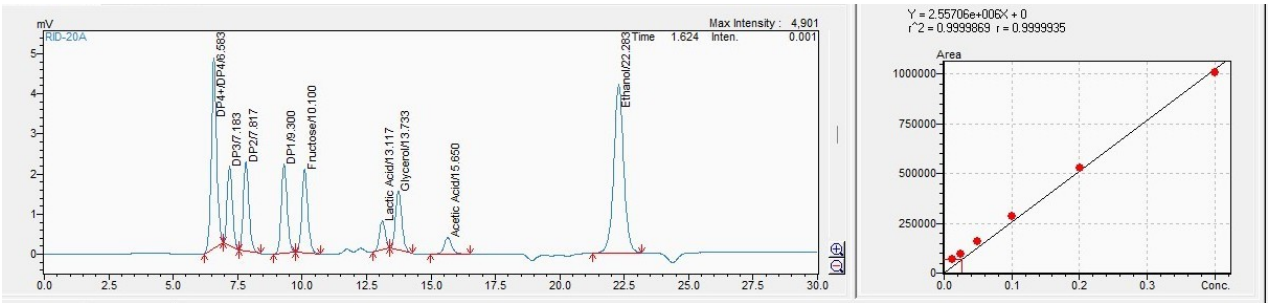

Figure 3.6: Calibration curve

The use of reliable standard solutions is critical for obtaining correct and reproducible results in chromatographic analysis.

The fermentation samples were analysed for a range of compounds, including sugars, oligosaccharides (DP fractions), fermentation products, and organic acids. These analytes were selected to provide a comprehensive understanding of fermentation behaviour and efficiency.

Sugars such as glucose, fructose, and sucrose serve as primary substrates for yeast metabolism. Oligosaccharides (DP1–DP4) represent different degrees of polymerization and provide insight into the breakdown of complex carbohydrates during fermentation.

Fermentation products such as ethanol show the efficiency of the fermentation process, while by-products such as glycerol reflect metabolic activity. Organic acids such as acetic acid and lactic acid can show process conditions and contamination.

3.6 Analytes Studied in Fermentation Samples

A variety of substances, such as sugars, oligosaccharides (DP fractions), fermentation products, and organic acids, were examined in the fermentation samples. These analytes were chosen to offer a thorough comprehension of the efficiency and behaviour of fermentation.

The main substrates for yeast metabolism are sugars like glucose, fructose, and sucrose. Various levels of polymerization are represented by oligosaccharides (DP1–DP4), which shed light on how complex carbohydrates are broken down during fermentation.

While byproducts like glycerol show metabolic activity, fermentation products like ethanol show how well the fermentation process is working. Acetic and lactic acids are examples of organic acids that might reveal contamination and process conditions.

Category	Analytes	Description
Sugars	Sucrose, glucose, and fructose	Primary substrates
Oligosaccharides	DP1, DP2, DP3, DP4	Polymerization levels
Alcohols	Ethanol	Main product
By-products	Glycerol	Secondary metabolite
Organic Acids	Acetic acid, lactic acid	Fermentation indicators

Table 3.3: Analytes Studied in Fermentation Samples

3.7 Sample Preparation

Sample preparation was conducted according to laboratory SOPs to ensure consistency, accuracy, and suitability of samples for chromatographic analysis. Separate preparation procedures were followed for molasses-based and grain-based fermentation samples due to differences in composition and concentration.

3.7.1 Preparation of Molasses Samples

Prevent contamination, samples of molasses were gathered in dry, clean beakers. After proper taring, a known amount of sample (1 g) was precisely weighed using an analytical balance. In order to obtain the proper dilution for HPLC analysis, the sample was then diluted with mobile phase to a final weight of 100 g.

Guarantee total homogenization, the diluted material was carefully stirred using a magnetic stirrer on a hot plate. Until a homogeneous solution was achieved, constant stirring was continued.

In order to cut suspended particles and avoid column blockage, the sample was filtered using a syringe filter (0.2 µm) after mixing. After filtering, the material was put into labelled HPLC vials for examination.

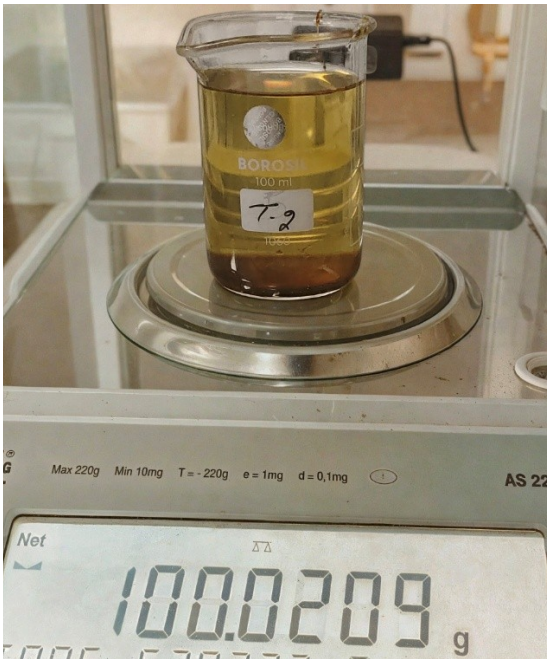

Figure 3.7: Preparation of molasses sample dilution using analytical balance.

Molasses samples were diluted gravimetrically by weighing the required quantity of sample and mobile phase using an analytical balance. Final dilution weight was adjusted to approximately 100 g before further processing.

3.7.2 Preparation of Grain-Based Fermentation Samples

Samples of fermented grain-based wash were gathered in sterile Eppendorf tubes. A fixed dilution strategy was used because of the increased turbidity and complexity of the matrix.

Using a calibrated micropipette, 0.1 mL of the sample was added to the Eppendorf tube after an aliquot of 1.0 mL mobile phase, creating an 11-fold dilution.

Guarantee adequate mixing and even analyte dispersion, the liquid was thoroughly vortexed. A syringe filter (0.2 µm, 13 mm diameter) was then used to filter the diluted sample in order to cut small particles. The filtrate was gathered and appropriately labelled in HPLC vials.

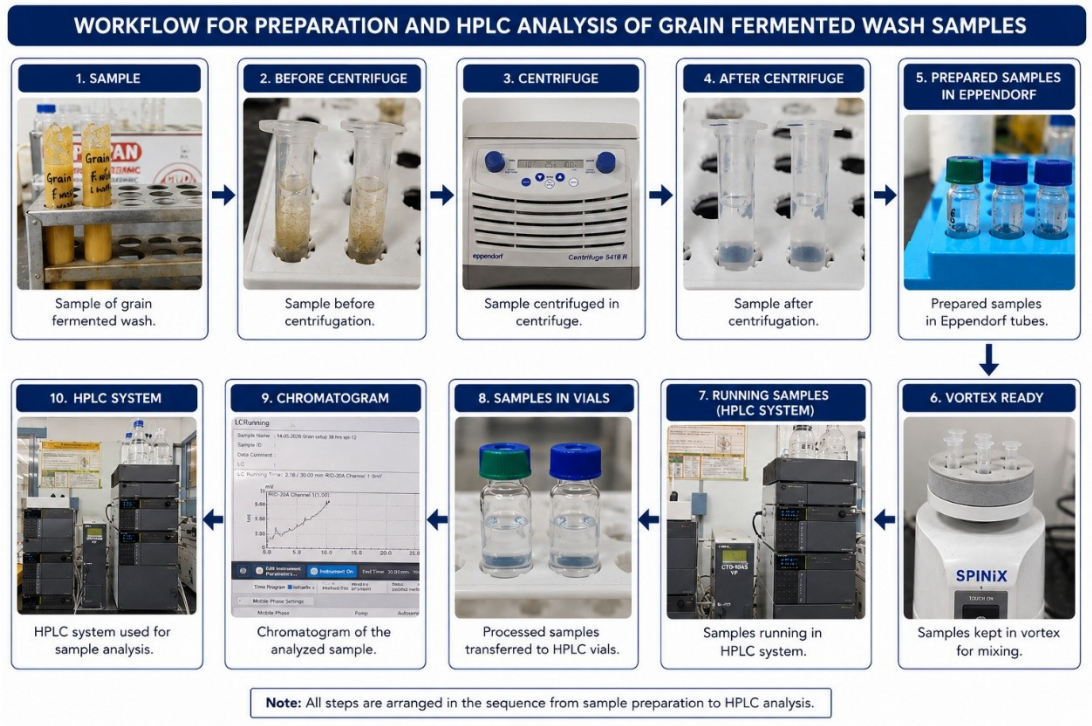
WORKFLOW FOR PREPARATION AND HPLC ANALYSIS OF GRAIN FERMENTED WASH SAMPLES
1. SAMPLE
Sample of grain fermented wash.
2. BEFORE CENTRIFUGE
Sample before centrifugation.
3. CENTRIFUGE
Sample centrifuged in centrifuge.
4. AFTER CENTRIFUGE
Sample after centrifugation.
5. PREPARED SAMPLES IN EPPENDORF
Prepared samples in Eppendorf tubes.
6. VORTEX READY
Samples kept in vortex for mixing.
7. RUNNING SAMPLES (HPLC SYSTEM)
Samples running in HPLC system.
8. SAMPLES IN VIALS
Processed samples transferred to HPLC vials.
9. CHROMATOGRAM
Chromatogram of the analyzed sample.
10. HPLC SYSTEM
HPLC system used for sample analysis.
Note: All steps are arranged in the sequence from sample preparation to HPLC analysis.

Figure 3.8 Workflow for Preparation and HPLC Analysis of Grain-Based Fermentation Samples

The figure illustrates the sequential workflow followed for the preparation and chromatographic analysis of grain-based fermented wash samples. The collected samples were diluted using mobile phase, vortex mixed for homogenization, centrifuged to remove suspended particles, and the clear supernatant was transferred into HPLC vials for analysis. The processed samples were subsequently analysed using the HPLC-RID system to obtain chromatographic profiles of the fermentation samples.

3.7.3 Filtration and Sample Clarity

In order to cut suspended particles and enhance chromatographic clarity prior to HPLC analysis, filtration was an essential stage in sample preparation. Before being injected into the chromatographic apparatus, the fermentation samples were filtered using 0.2 µm syringe membrane filters after being diluted and mixed.

During repeated analytical runs, this filtration process protected the chromatographic column from contamination or obstruction, reduced particle interference, and kept a steady baseline response. Additionally, during chromatographic analysis, proper filtration enhanced peak quality and reproducibility.

Before being transferred into HPLC vials, fermentation samples based on molasses and grains were meticulously filtered. Following filtration, the clear filtrate was used for chromatographic analysis.

Figure 3.9: Syringe filtration of fermentation sample using a 0.2 µm syringe filter.

The fermentation sample was filtered prior to HPLC analysis to remove suspended particles and improve chromatographic performance.

3.7.4 HPLC Injection

Prepared samples were analysed using an HPLC system after proper filtration. Blank and standard solutions were run prior to sample injection to ensure system suitability and calibration.

- Molasses samples were analysed using an Agilent Hi-Plex H column.
- Grain-based samples were analysed using a Bio-Rad Amine column.

3.8 Chromatographic Conditions

High-performance liquid chromatography (HPLC) was used for chromatographic analysis to separate and measure the samples' sugars, oligosaccharides, and fermentation byproducts.

The kind of sample matrix and the physicochemical characteristics of the analytes were taken into consideration when optimizing the operating parameters.

Depending on the type of sample, two distinct chromatographic columns were employed to conduct effective separation and precise detection.

3.8.1 Chromatographic Conditions for Molasses Samples

An Agilent Hi-Plex H column, which is specifically designed for the separation of organic acids, sugars, and alcohols under acidic conditions, was used to evaluate molasses samples.

Diluted sulfuric acid (0.005 M) was used as the mobile phase, which enhances peak resolution and makes ion-exclusion separation easier. A regulated flow rate of 0.6 mL/min was used for the study.

In order to improve separation efficiency and lower the viscosity of the mobile phase, the column temperature was kept at 60°C. A Refractive Index Detector (RID) was employed, and it was run at 50°C to preserve baseline stability.

Maintain uniformity, the injection volume was set at 20 µL for every sample and standard.

Parameter	Condition
Instrument	Shimadzu HPLC 1220
Detector	Refractive Index Detector (RID)
Mobile phase	0.005 M H_2SO_4
Flow rate	0.5 mL/min
Injection volume	20 µL
Column temperature	35°C
Detector temperature	35°C

Table 3.4: Chromatographic Conditions for Molasses Samples

The chromatographic conditions were kept throughout the analysis to obtain stable separation and reproducible detector response. The use of 0.005 M H_2SO_4 as the mobile phase supported the separation of sugars, organic acids, ethanol, and other fermentation-related metabolites using RID detection.

3.8.2 Chromatographic Conditions for Grain-Based Samples

A Bio-Rad Amine column, which is proper for the investigation of fermentation metabolites and carbohydrates, was used to examine grain-based fermentation samples.

Although operating conditions were significantly altered to accommodate the grain matrix, the same mobile phase (5 mM sulfuric acid) was used.

Both the RID detector temperature and the column temperature were kept at 35°C. Ensure comparability among analyses, the injection volume (20 µL) and flow rate (0.6 mL/min) were kept.

Parameter	Condition
Instrument	Shimadzu HPLC 1220
Detector	Refractive Index Detector (RID)
Mobile phase	0.005 M H_2SO_4
Flow rate	0.6 mL/min

Injection volume	20 µL
Column temperature	60°C
Detector temperature	50°C

Table 3.5: Chromatographic Conditions for Grain-Based Samples

The chromatographic conditions were maintained throughout the analysis to obtain stable separation and reproducible detector response. The use of 0.005 M H_2SO_4 as the mobile phase supported the separation of sugars, organic acids, ethanol and other fermentation-related metabolites using RID detection. The column and detector temperatures were controlled to maintain stable retention behaviour and

3.9 Analytical Procedure

The HPLC system was filled with prepared samples and reference solutions. Analytes were separated according to how they interacted with the stationary phase and mobile phase.

The retention time of each compound was used to find it, and peak area from chromatograms was used to quantify it.

3.10 Calibration and Quantification

Quantitative analysis was performed using calibration curves prepared from standard solutions. The concentration of analytes in the samples was calculated by comparing peak areas with standard calibration curves.

3.11 Monitoring of Fermentation

Fermentation samples were analysed at different time intervals to see changes in sugar concentration and formation of fermentation products such as ethanol and organic acids. This helped in understanding fermentation behaviour and efficiency.

3.12 Data Analysis

The chromatographic data obtained were analysed to decide sugar consumption, formation of metabolites, and overall fermentation performance. Results were interpreted based on retention time and peak area.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Chromatographic Profiling of Fermentation Samples

Different chromatographic patterns related to sugars, oligosaccharides, alcohols, and organic acids produced during microbial fermentation were seen in the fermentation samples examined during the investigation.

Under ideal chromatographic circumstances, High Performance Liquid Chromatography (HPLC) analysis successfully separated the chemicals found in both grain-based and molasses-based fermentation samples. Throughout the analytical runs, stable baseline response and adequate peak separation were seen, proving the chromatographic system's appropproper performance Hout sample analysis.

The substances created or consumed throughout the fermentation process were represented by several peaks that appeared at various retention durations in the chromatograms derived from the examined samples. The chromatographic profile showed strong major peaks that corresponded to ethanol and oligosaccharides. During the study, smaller peaks that corresponded to organic acids and intermediary metabolites were also found. The continuous metabolic activity in the fermentation system was reflected in these changes in the chromatographic peak pattern.

The samples' chromatographic activity showed that carbohydrates were being converted into fermentation metabolites by microbes. During fermentation, bacteria, yeast, and related microbial communities use carbohydrates to produce ethanol, glycerol, and organic acids. Through variations in peak strength and chemical distribution, the HPLC chromatograms bought during the investigation amply deprovedhese metabolic shifts.

The chromatographic profiles of samples based on molasses and grain differed noticeably. After dilution and filtering, samples based on grain yielded somewhat cleaner chromatograms, but samples based on molasses had more complex organic components and deeper pigmentation. These variations are linked to changes in the concentration of dissolved particles in the fermentation matrix and the composition of the substrate.

Additionally, the chromatographic analysis showed how crucial it is to properly prepare the material prior to HPLC injection. Chromatographic clarity was much enhanced by filtration and dilution, which also avoided

substantial baseline disturbance. The dependability of the analytical technique used during the investigation was confirmed by stable chromatographic conditions across many injections.

Blank injections were first conducted during the analytical sequence to verify baseline stability and system cleanliness. After that, standard solutions were injected to compare retention times and find analytes. Ensure analytical consistency and track system appropriateness, fermentation samples with prepared standard injection, and standards were reinjected every five samples.

Vital details about the course of fermentation and substrate usage were revealed by the chromatographic profiles produced during the investigation. The conversion of carbohydrates into fermentation metabolites was shown by changes in chromatographic peak strength. Active microbial fermentation within the examined samples was shown by the formation of ethanol and organic acid peaks together with a decrease in sugar-related peaks.

The study showed that because HPLC analysis enables the simultaneous detection of several substances within a single analytical run, it is especially useful for monitoring fermentation systems. The study's use of the chromatographic approach made it possible to properly characterize fermentation samples and assess the biochemical changes that took place throughout fermentation.

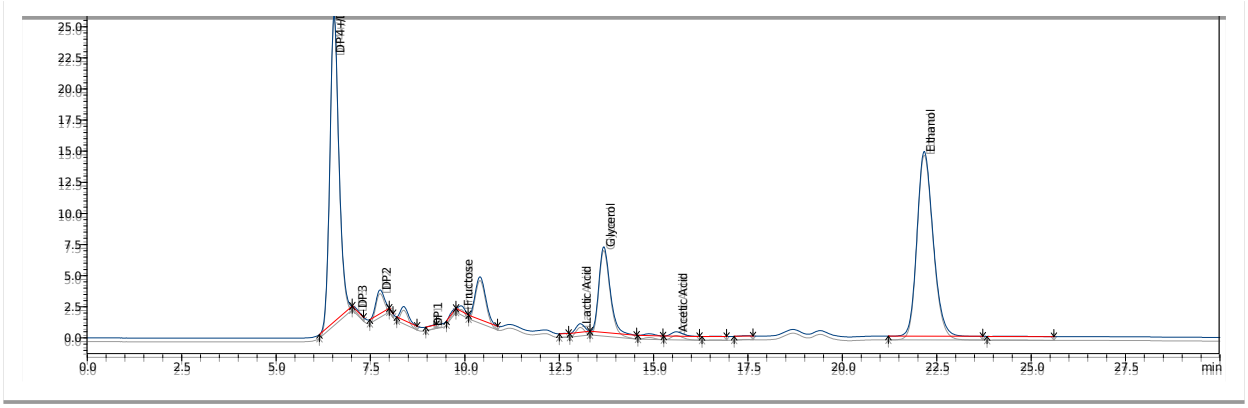

Figure 4.1: HPLC chromatogram showing separation of sugars and fermentation metabolites.
The chromatogram demonstrated clear peak development and efficient separation of fermentation metabolites. Ethanol was represented by a large peak, whilst sugars and intermediate fermentation products were represented by smaller peaks.

4.2 Changes in Sugar Composition During Fermentation

The fermentable sugars and oligosaccharide fractions found in the fermentation samples were clearly separated by chromatographic examination. DP4+/DP4, DP3, DP2, DP1, and fructose were among the substances found during the analysis. Retention behaviour from standard chromatograms examined under the same chromatographic conditions was used to find these substances.

The existence of partially hydrolysed carbohydrate molecules throughout the fermentation process was suggested by the presence of distinct DP fractions. In the chromatographic profile, the DP4+/DP4 fraction displayed a peak intensity that was significantly higher than that of other DP fractions.

Additionally, smaller peaks corresponding to DP3, DP2, and DP1 were seen, suggesting that bigger carbohydrate molecules gradually broke down into smaller oligosaccharides and fermentable sugars.

The enzymatic and microbiological conversion that takes place during fermentation is reflected in the reported chromatographic profile. Complex carbohydrates found in grain-based substrates are hydrolysed during industrial fermentation, producing smaller sugar molecules that microbes can use to produce energy and metabolites.

Simple sugar peaks in fermented samples were less intense, showing that fermentable sugars were actively used during microbial metabolism. Since sugars serve as the main substrate for the creation of ethanol, a

decrease in sugar concentration is thought to be a significant signal of fermentation progress.

During examination, fructose was found as a clear chromatographic peak. The existence and intensity of fructose peaks differed between samples, suggesting variations for sugar used and usedosition of the substrate. Differences in microbial activity and fermentation efficiency were reflected in variations in sugar peak intensity among fermentation samples.

The chromatographic separation achieved during analysis showed that the chromatographic columns used in the investigation had an acceptable resolving capability. Because there was little peak overlap, the carbohydrate fractions could be correctly found. Repeated injections showed stable retention behaviour, suggesting acceptable chromatographic repeatability.

During the study, fructose was found as a clear chromatographic peak. The existence and intensity of fructose peaks differed between samples, suggesting variations for sugar used and the composition of the substrate. Differences in microbial activity and fermentation efficiency were reflected in variations in sugar peak intensity among fermentation samples.

The chromatographic separation that was achieved during analysis showed that the chromatographic columns used in the investigation had a reasonable resolving capability. Because there was little peak overlap, the fractions of carbohydrates could be correctly found. Repeated injections showed stable retention behaviour, showing acceptable chromatographic reproducibility.

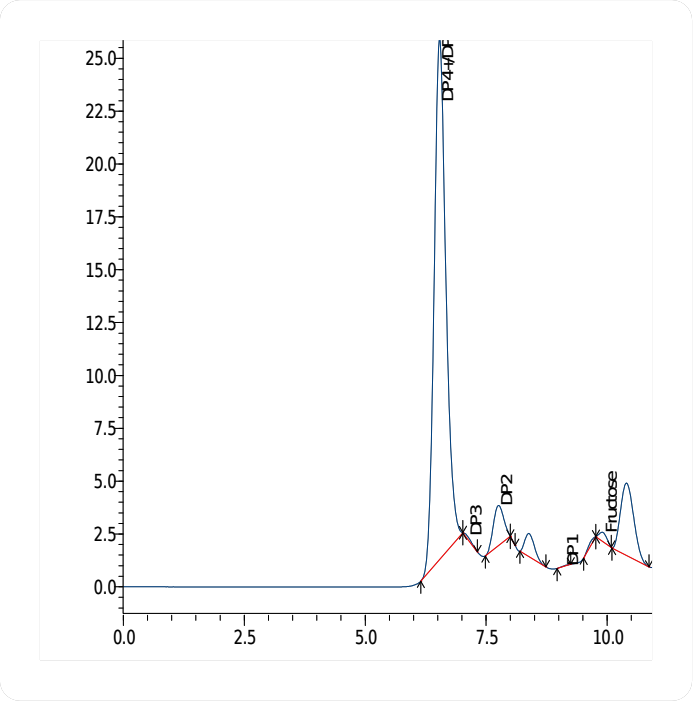

Figure 4.2: Separation of DP fractions and sugars in the fermentation sample

Distinct peaks corresponding to DP fractions and sugars were seen during chromatographic analysis with satisfactory separation and minimal peak overlapping.

4.3 Detection of Fermentation Byproducts

The development of significant fermentation metabolites, such as ethanol, glycerol, lactic acid, and acetic acid, was verified by chromatographic analysis in the examined fermentation samples. Based on their retention behaviour and comparison with reference chromatograms examined under the same chromatographic

circumstances, these substances were successfully separated and found during HPLC analysis.

Ethanol had the strongest and most noticeable chromatographic peak of all the metabolites found, showing robust fermentation and effective microbial conversion of carbohydrates into alcohol. One of the main biochemical processes that takes place during fermentation is the production of ethanol, especially during yeast metabolism when fermentable carbohydrates are transformed into carbon dioxide and alcohol under the right environmental circumstances.

The development of significant fermentation metabolites, such as ethanol, glycerol, lactic acid, and acetic acid, in the examined fermentation samples was verified by chromatographic analysis. Based on their retention behaviour and comparison with reference chromatograms examined under identical chromatographic circumstances, these substances were successfully separated and found during HPLC analysis.

Ethanol had the strongest and most noticeable chromatographic peak of all the metabolites found, showing robust fermentation and effective microbial conversion of carbohydrates into alcohol. One of the main biochemical processes that take place during fermentation is the production of ethanol, especially during yeast metabolism, where fermentable carbohydrates are transformed into carbon dioxide and alcohol under the right environmental circumstances.

Organic acids such as lactic acid and acetic acid were also found in the chromatograms with excellent peak separation. These metabolites can affect the fermentation environment as a whole and are produced during microbial biochemical pathways. In comparison to ethanol, the considerably smaller peaks for organic acids showed lower amounts in the examined samples.

Grain-based and molasses-based fermentation samples showed different metabolite peak intensities. Variations in ethanol production and organic

acid distribution mirrored changes in substrate composition, microbial activity, and fermentation progression. Grain-based samples proved much clearer chromatographic profiles following dilution and filtration, whereawhileses-based samples containehadmplicated organic content.

The chromatographic study showed that the chosen HPLC settings were proper for tracking fermentation metabolites in samples of industrial fermentation. Throughout the analytical runs, there was no discernible peak overlap or baseline disruption, and the baseline response remained stable, and the peak resolution was excellent.

The study's findings showed that HPLC is a useful analytical method for tracking the metabolic alterations that take place during fermentation. When ethanol, glycerol, and organic acids are detected simultaneously in a single chromatographic run, vital details about microbial metabolism, substrate usage, and fermentation efficiency are revealed.

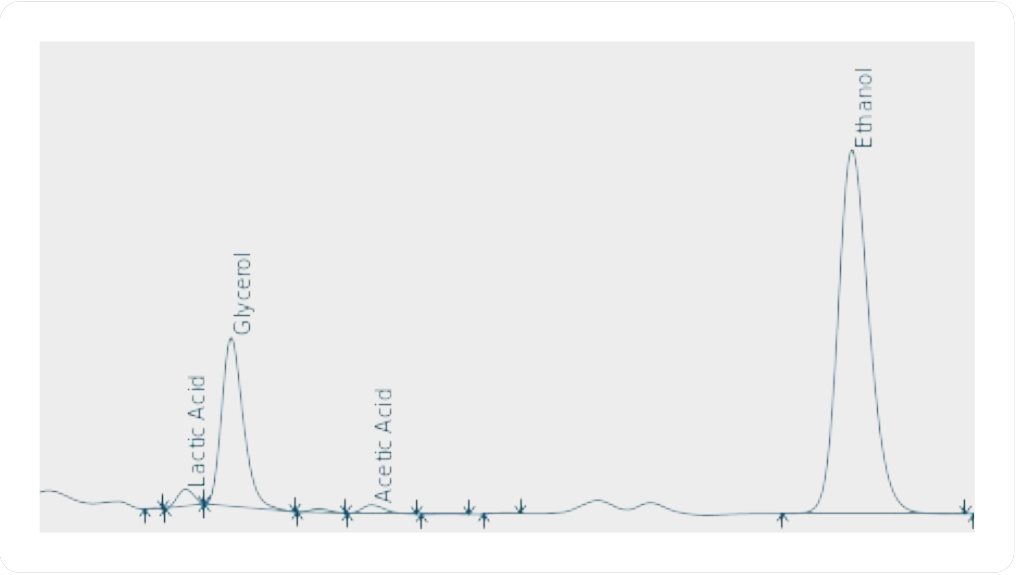

Figure 4.3: Detection of fermentation metabolites during HPLC analysis

Distinct peaks corresponding to ethanol, glycerol, lactic acid, and acetic acid were seen during chromatographic analysis. The comparatively higher ethanol peak intensity showed active fermentation and efficient conversion of sugars into fermentation products.

4.4 Time-Dependent Changes During Fermentation

A dynamic biological process, fermentation causes a constant change in the sample's composition over time. Consequently, the development of fermentation cannot be fully explained by the chromatographic profile bought at a single time point. In order to track changes in the main fermentable sugars and fermentation products, samples were taken at various fermentation intervals, including 0, 12, 24, and 36 hours.

Glucose, fructose, ethanol, glycerol, lactic acid, and acetic acid were the primary substances seen in the chromatograms. These substances are crucial markers of the generation of secondary metabolites, alcohol, and substrate use during fermentation.

The sample primarily reflected the first substrate composition prior to active microbial conversion during the first stages of fermentation, particularly at 0 h. At this stage, the chromatographic profile displayed peaks associated with sugar that were somewhat higher. These peaks, along with added peaks formed from carbohydrates depending on the raw material employed, were mostly linked to accessible fermentable sugars like glucose and fructose. Because starch-based substrates must be broken down into simpler sugars prior to effective fermentation, the first chromatogram of grain-based samples also displayed peaks associated with partially hydrolysed carbohydrate fractions. Because molasses already includes easily accessible sugars, the sugar profile in samples based on molasses was somewhat more straightforward. Consequently, the first sugar load of the fermentation medium was represented in the early-stage chromatogram.

A discernible decrease in the peak intensity associated with sugar was seen as fermentation advanced from 0 to 12 hours. This suggests that accessible sugars were being used by microbes for metabolic activity. The ethanol peak started to appear or rise at this phase, showing the transformation of glucose into alcohol. An essential indicator of active fermentation was the rise in the ethanol peak. In addition to ethanol,

slight variations in the peaks of glycerol and organic acid were also noted. While organic acids like lactic acid and acetic acid might result from microbial activity, substrate composition, or mixed fermentation circumstances, glycerol synthesis is often linked to yeast metabolism and stress balance during fermentation.

A discernible decrease in the peak intensity associated with sugar was noted as fermentation advanced from 0 to 12 hours. This suggests that microbes had begun using the carbohydrates that were accessible for metabolic activity. The ethanol peak, which shows the transformation of carbohydrates into alcohol, started to appear during this phase. An essential indicator of active fermentation was the rise in the ethanol peak. In addition to ethanol, slight variations in the peaks of organic acid and glycerol were also noted. While organic acids like lactic acid and acetic acid may result from microbial activity, substrate composition, or mixed fermentation circumstances, glycerol synthesis is often linked to yeast metabolism and stress balance during fermentation.

The chromatogram revealed a high ethanol peak and added reduction in sugar-related peaks at 36 hours. This pattern suggests that fermentation had progressed to a higher level.

The low strength of the fructose and glucose peaks showed that the fermenting microbes were effectively using the sugar. Alcohol generation was confirmed to be the primary result of the fermentation process by the ethanol peak's continued dominance. Additionally, glycerol was found, which could be connected to osmotic balance and yeast adaptability during fermentation. Although secondary fermentation products were generated, they did not dominate the chromatographic profile, as seen by the lesser amounts of organic acids.

Overall, it was clear from the time-dependent chromatographic changes that carbohydrates were converted into ethanol and other metabolites. The progress of fermentation was set up by the decrease in sugar peaks

and the increase in ethanol peaks with fermentation time. The fermentation system's metabolic behaviour was further revealed by the production of glycerol, lactic acid, and acetic acid. The results also proved that HPLC-RID's ability to separate and identifinds, alcohols, and organic acids in a single analytical run made it appropriatproper forg fermentation.

The most important observation from the time-based analysis was that the fermentation process did not still are chemically constant. The sample composition changed gradually from a sugar-rich profile at the beginning to an ethanol-dominant profile at later stages. This confirms that the microorganisms actively consumed the available carbon sources and converted them into fermentation products. Such time-based monitoring is useful because it helps in understanding the stage of fermentation, efficiency of sugar use, and possible accumulation of by-products.

Later-stage samples in the current investigation had relatively low sugar peaks, showing that the fermentation was successful. However, precise quantification needs peak area computation and calibration using established standards. As a result, peak comparison and relative chromatographic response serve as the primary foundation for the current interpretation. Because variations in peak intensity and retention pattern can unmistakably show substrate consumption and metabolite synthesis, this method still yields valuable information about fermentation behaviour.

Fermentation Time	Sugar Peaks	Ethanol Peak	Glycerol Peak	Organic Acid Peaks	Interpretation
0 h	High	Absent/ Low	Low	Low	Initial substrate stage with available sugars
12 h	Decreasing	Increasing	Low to moderate	Low	Active fermentation started
24 h	Further reduced	High	Moderate	Low to moderate	Strong sugar conversion and ethanol formation
36 hr	Low	Dominant	Present	Present	Advanced

				at a low level	fermentation with effective sugar use

Table 4.1 General trend of major compounds during fermentation

4.5 Comparative Analysis of Grain-Based and Molasses-Based Samples

The chromatographic analysis of fermentation samples based on grain and molasses revealed distinct variations in metabolite production and sugar use. The type of raw materials used for fermentation was the primary cause of these variations. While molasses-based fermentation has easily accessible sugars that microbes can use directly, grain-based fermentation mostly relies on the conversion of complex carbohydrates into simple fermentable sugars. Both sample types displayed distinct chromatographic patterns as a result of this distinction.

The chromatograms of grain-based fermentation samples revealed several early peaks associated with components produced from carbohydrates. Hydrolysed starch fractions, glucose, maltose-like sugars, or other soluble carbohydrate components generated during fermentation may be linked to these peaks. Since starch is the primary source of carbohydrates in grains, sugars must first undergo enzymatic or microbiological hydrolysis before they can be fermented. This could account for the early retention region's several sugar-related peaks. These peaks decreased as fermentation went on, showing that microbes were using the available carbohydrates.

The chromatographic profile of fermentation samples based on molasses revealed a more straightforward pattern of sugar-to-ethanol conversion. Fermentable sugars, primarily sucrose, glucose, and fructose, are abundant in molasses. As a result, these sugars are easier for microbes to use than substrates based on grains.

In molasses samples, the emergence of an ethanol peak and a decrease in sugar peaks showed effective fermentation. One of the chromatogram's most noticeable peaks was the ethanol peak. This suggests that molasses served as a proper carbon source for the synthesis of alcohol.

In both kinds of samples, the most significant marker was the ethanol peak. The successful fermentation and conversion of carbohydrates into alcohol was shown by a prominent ethanol peak. Ethanol appeared as a prominent peak at a later retention time in the comparative chromatogram. Both grain and molasses samples had ethanol, which demonsprovedfermentation was taking place in both systems. However, depending on the substrate composition, fermentation duration, microbial activity, and sample processing, the relative intensities of the sugar and ethanol peaks may vary.

In the chromatographic profile, glycerol was also found. Because glycerol is often created as a secondary metabolite during yeast fermentation, its presence is important. It supports yeast cells' ability to withstand osmotic stress and keep redox balance. Despite not being the primary byproduct of alcoholic fermentation, glycerol detection offers valuable insights on microbial metabolism. Along with ethanol and organic acids, the glycerol peak was seen, showing that the fermentation system produced more than just ethanol.

The chromatograms also showed organic acids like acetic and lactic acid. Because they show side metabolic activity during fermentation, these acids are significant. Acetic acid can be created by oxidation or bacterial activity, while lactic acid can be formed by lactic acid bacteria or by secondary metabolic processes. These acids are often found in smaller concentrations during controlled fermentation. The organic acid peaks in the current chromatograms were significantly smaller than the ethanol peak, showing that the primary fermentation result was ethanol generation.

Although both substrates allowed fermentation, the chromatographic behaviour of the grain and molasses fermentation samples differed. Due to the presence of suspended matrix components and chemicals generated from starch, grain-based samples displayed a more complicated profile. Because there were soluble sugars in the molasses-based samples, the sugar and ethanol-related peaks were more visible. This distinction emphasizes how crucial substrate type is to fermentation analysis.

The clarity of the sample was another significant finding. Because of the insoluble particles and suspended solids, grain-based samples were more turbid. For grain samples, centrifugation and filtration were crucial prior to HPLC injection. Improved peak separation and a steady baseline were made possible by better sample clarity. Despite being liquid, molasses samples needed to be diluted and filtered due to their dark colour and high viscosity. For both kinds of samples, proper sample preparation was needed to safeguard the column and enhance chromatographic quality.

Overall, the comparative chromatographic analysis verified that HPLC-RID was useful for analysing fermentation materials based on molasses and grains. Major sugars, ethanol, glycerol, and organic acids may all be detected with this approach in a single run. The fermentation process was aided by the formation of an ethanol peak and the decrease in sugar peaks. The two substrates' physicochemical characteristics and carbohydrate makeup were the primary causes of their variances.

Parameter	Grain Fermentation Sample	Molasses Fermentation Sample
Substrate Type	Starch-based substrate	Sucrose-rich substrate
Initial Sugar Composition	Complex carbohydrates and oligosaccharides	High sucrose concentration
Sample Appearance	Turbid yellow suspension	Dark brown viscous liquid
Fermentation Behaviour	Gradual sugar hydrolysis and ethanol formation	Rapid sugar utilization and ethanol production

Ethanol Peak Intensity	Moderate to high	High
Organic Acid Formation	Moderate	Moderate to high
Presence of DP Fractions	Clearly observed	Less prominent
Sample Preparation Requirement	Centrifugation and filtration required	Dilution and filtration required
Chromatographic Complexity	Multiple peaks observed	Relatively simpler peak profile
Industrial Relevance	Grain alcohol production	Molasses-based distillery fermentation

Table 4.2 Comparison of grain and molasses fermentation samples

The comparative analysis showed clear differences between grain-based and molasses-based fermentation samples. Grain samples displayed more complex carbohydrate-related peaks, while molasses samples showed stronger ethanol response due to the presence of readily fermentable sugars. The chromatographic profiles confirmed active fermentation behaviour in both substrates under the applied HPLC-RID conditions.

4.6 Fermentation Monitoring Through HPLC Analysis

By analysing sugars and fermentation metabolites, the HPLC approach used in the study proved efficacy in tracking the process of fermentation. The metabolic changes that take place during the microbial conversion of carbohydrates into alcohols and organic acids might be seen thanks to sequential chromatographic analysis.

The creation of ethanol peaks in fermented samples together with a decrease in sugar-related peaks was one of the study's key findings. This chromatographic behaviour proved that bacteria actively used fermentable carbohydrates during fermentation. Progressive substrate conversion and metabolic activity were confirmed by lower sugar content and higher ethanol peak intensity.

Additionally, the chromatographic examination revealed the production of secondary metabolites, such as acetic acid, lactic acid, and glycerol.

These substances are linked to microbial metabolic pathways and offer valuable insights into the conditions of fermentation and the biochemical activity taking place in the system.

All of the analytical runs were conducted under stable chromatographic conditions. Prior to standard and sample analysis, blank injections proved adequate baseline stability without significant interfering peaks. Every five sample injections, standard solutions were reinjected to preserve analytical consistency and track chromatographic reproducibility.

Achieving adequate chromatographic performance was largely dependent on proper sample preparation. Centrifugation, filtering, vortex mixing, and dilution greatly enhanced chromatographic clarity and decreased particle interference during analysis. During chromatographic runs, these pretreatment procedures helped ensure a steady baseline response and efficient peak separation.

In a single analytical run, several chemicals related to fermentation systems might be simultaneously detected using the chromatographic technique. Because of this, HPLC is a very practical analytical method for routine industrial fermentation process checking and metabolite characterization.

The study's findings proved that HPLC analysis can offer comprehensive insight into the development of metabolites, substrate consumption, and fermentation. Microbial activity and overall fermentation efficiency can be assessed by tracking chromatographic changes during fermentation.

The study showed that chromatographic analysis may be used successfully for routine fermentation analysis and is especially useful for tracking biochemical changes that take place throughout industrial fermentation operations.

4.7 Interpretation of Major Fermentation Metabolites

The fermented samples' HPLC-RID chromatograms revealed the presence of significant fermentation-related substances like organic acids, ethanol, glycerol, and leftover sugars. These substances have a direct bearing on how well fermentation proceeds. While the production of ethanol and glycerol shows active microbial metabolism, the presence or decrease of sugar peaks provides information about substrate use.

The sugar-related peaks in the chromatographic profile were mostly seen in the chromatogram's earlier section. Depending on the typical retention duration, these peaks could show fructose, glucose, and other carbohydrate components. These sugar peaks were less intense in fermented samples than in the expected first sugar-rich period. This implies that fermentation used a significant amount of fermentable carbohydrates. Better conversion of carbohydrates into fermentation products is shown by low sugar peaks in the later fermentation samples.

The most significant byproduct found in the fermented samples was ethanol. The chromatogram revealed a distinct and prominent ethanol peak, showing the effective completion of alcoholic fermentation. The fact that the ethanol peak was more noticeable than the sugar peaks suggests that the available sugars were fermented into ethanol. The basic metabolic process of fermentation, in which yeast or fermenting microbes use carbohydrates and generate ethanol as the primary end product, is supported by this study.

In the fermented samples, ethanol was the most significant product. Alcoholic fermentation had been effective because the chromatogram showed a distinct and prominent ethanol peak. Since the ethanol peak was more noticeable than the sugar peaks, fermentation produced ethanol from the available sugars. This outcome confirms the fundamental biochemical process of fermentation, in which yeast or fermenting microbes use carbohydrates to generate ethanol as the primary byproduct.

In the chromatographic profile, glycerol was also found. One typical secondary metabolite generated during yeast fermentation is glycerol. Its

existence during fermentation is a sign of active yeast metabolism and cellular balance. Glycerol synthesis is significant because it is the physiological activity of fermenting bacteria, even if ethanol is the primary result. Both main and minor fermentation metabolites could be detected using the HPLC-RID approach, as seen by the glycerol peak's obvious separation from the ethanol peak.

The general metabolite pattern shows that ethanol generation was the primary goal of the fermentation process. Active fermentation is shown by the strong ethanol peak, evidclearcerol peak, and low sugar response. Minor organic acid peaks show that there was some side metabolic activity, which is typical in commercial or natural fermentation systems.

Compound	Compound Type	Observation	Scientific Interpretation
Glucose	Fermentable sugar	Reduced with fermentation time	Used by microorganisms as a carbon source
Fructose	Fermentable sugar	Reduced with fermentation time	Consumed during fermentation
Ethanol	Alcohol	Increased/ dominant at a later stage	Main product of alcoholic fermentation
Glycerol	Polyol	Detected in samples	Indicates yeast metabolism and stress balance
Lactic acid	Organic acid	Small peak	May show lactic acid bacterial activity or side pathway
Acetic acid	Organic acid	Small peak	May show oxidation or side fermentation activity

Table 4.3: Interpretation of major fermentation metabolites detected by HPLC-RID

The metabolite profile supports the occurrence of alcoholic fermentation. Ethanol was the major fermentation product, while glycerol and organic acids appeared as secondary metabolites. The decrease in fermentable sugars and increase in ethanol response are the strongest indicators of fermentation progress.

4.8 Analytical Stability and Reliability of HPLC-RID Method

Proper sample preparation, a steady baseline, distinct peak separation, and a reproducible standard response are all necessary for the accuracy of chromatographic results. In this investigation, sugars, ethanol, glycerol, and organic acids were found in fermentation samples using HPLC-RID analysis. The chromatograms proved that the chosen technique was appropproper foring the significant alterations taking place throughout fermentation.

Prior to sample analysis, the blank chromatogram was used to verify baseline conditions and system cleanliness. Major interfering peaks in the retention areas of significant compounds were absent from the blank run. This verified that the system and mobile phase were suitable for added examination. Because contamination or carryover can produce misleading peaks and interfere with the interpretation of sample results, a reliable blank is crucial.

For significant chemicals, the standard chromatogram had distinct peaks. Identification of the substances in the sample chromatograms was aided by the separation of sugar-related compounds, glycerol, organic acids, and ethanol. The sample peaks were compared using the standards' retention pattern as a guide. Because the compounds were recognized by comparison with typical retention behaviour rather than only by assumption, the interpretation became more trustworthy.

Important chemicals had distinct peaks in the standard chromatogram. The identification of the chemicals in the sample chromatograms was aided by the separation of sugar-related compounds, ethanol, glycerol, and organic acids. The sample peaks were compared using the standards' retention pattern as a reference. Because the compounds were recognized by comparison with typical retention behaviour rather than only by assumption, the interpretation became more trustworthy.

The principal peaks in the sample chromatograms were properly separated. The peaks of ethanol, glycerol, and organic acid were sufficiently distinct to be interpreted. This suggests that the resolution of the approach was suitable for monitoring fermentation. Stable temperature, mobile phase flow, and proper sample clarity are crucial for RID-based analysis. Any suspended particle or strongly coloured impurity has the potential to alter the detector response or upset the baseline. As a result, centrifugation and filtering were crucial in raising the analysis's dependability.

The principal peaks were properly separated in the sample chromatograms. The peaks of ethanol, glycerol, and organic acid were sufficiently separated for interpretation. This suggests that the method's resolution was suitable for monitoring fermentation. Stable temperature, mobile phase flow, and proper sample clarity are critical in RID-based analysis. The baseline may be disturbed, or the detector response may be affected by any suspended particle or brightly coloured contaminant. As a result, centrifugation and filtration were crucial in raising the accuracy of the analysis.

Parameter	Observation	Meaning
Standard injection after sample sequence	Stable/acceptable	The method response remained dependable.
Retention time	Consistent	Compound identification was dependable.
Peak separation	Properly separated	The method was suitable for sugars, alcohols, and organic acids.
Baseline	Stable	The detector and mobile phase conditions were acceptable.
Sample clarity	Improved after centrifugation and filtration	Reduced risk of column blockage and poor peak shape
Overall method performance	Acceptable	The HPLC-RID method was suitable for

		fermentation monitoring.

Table 4.4: Analytical stability and reliability observations of the HPLC-RID method

The method showed acceptable reliability for monitoring fermentation samples. The blank run confirmed system cleanliness, the standard run supported compound identification, and the sample runs showed clear detection of fermentation metabolites. Since the standard response remained stable after sample analysis, the analytical sequence was considered dependable.

4.9 Overall Discussion of Fermentation Efficiency

By examining the connection between sugar use and the production of fermentation products, fermentation efficiency was assessed. Microorganisms steadily consume fermentable sugars during a successful fermentation process, converting them into principal products like ethanol as well as secondary metabolites like glycerol and organic acids. The chromatographic observations in the current investigation proved this antic expected, with the ethanol response being clearly visible and sugar-related responses being relatively decreased.

The decrease in sugar-related peaks shows that the available fermentable sugars were using microbial metabolism. Sugars such as glucose and fructose function as primary carbon sources for yeast and other fermenting microorganisms. As fermentation progresses, these sugars are consumed and converted into ethanol through alcoholic fermentation.

Therefore, lower sugar response in fermented samples is an important sign of substrate uses the main fermentation products was found to be ethanol. Strong ethanol responses show that the fermentation process was vigorous and that alcohol was produced from the available carbohydrates. One of the most crucial signs of a good alcoholic

fermentation process is this. The conclusion that fermentation went ahead well is supported by the presence of ethanol and a decreased sugar response.

The fermentation samples also had glycerol. Despite not being the primary byproduct of alcoholic fermentation, the presence of glycerol is significant since it signifies ongoing yeast metabolism. Glycerol is a secondary metabolite produced by yeast cells during fermentation, mostly to keep cellular equilibrium in the face of fermentation stress. Thus, the existence of active microbial metabolism is supported by the detection of glycerol.

One of the main products of fermentation was found to be ethanol. Strong ethanol production shows that the fermentation process was vigorous and that alcohol was produced from the available carbohydrates. One of the most crucial markers of a good alcoholic fermentation process is this. The conclusion that fermentation went ahead well is supported by the presence of ethanol and the decreased sugar response.

In the fermentation samples, glycerol was also found. The presence of glycerol is significant because it shows active yeast metabolism, even though it is not the primary byproduct of alcoholic fermentation. Glycerol is a secondary metabolite that yeast cells make during fermentation, mostly to preserve cellular equilibrium in the face of fermentation stress. Thus, the existence of active microbial metabolism is supported by glycerol detection.

One of the main fermentation products was noted to be ethanol. The fermentation process was vigorous, and the available sugars were transformed into alcohol when there was a high ethanol reaction. This is one of the most crucial signs that alcoholic fermentation is going well. The presence of ethanol and a decreased sugar response lend credence to the idea that fermentation went ahead successfully.

Additionally, glycerol was found in the fermentation samples. Glycerol is a byproduct of alcoholic fermentation, but its presence is significant since it shows that yeast metabolism is occurring.

Glycerol is a secondary metabolite that yeast cells make during fermentation, mostly to preserve cellular equilibrium in the face of fermentation stress. Glycerol measurement thus confirms the existence of active microbial metabolism.

Indicator	Observation	Conclusion
Sugar use	Sugar peaks decreased with time.	Fermentation consumed available sugars.
Ethanol formation	Ethanol peak increased and became dominant.	Alcoholic fermentation was successful.
Glycerol formation	Glycerol peak detected	Secondary yeast metabolism occurred.
Organic acid formation	Lactic and acetic acid peaks were detected at a lower level.	Side metabolites were formed but did not dominate.
Substrate comparison	Grain and molasses showed different profiles.	Raw material strongly influenced the chromatographic pattern.
Overall result	Low residual sugars and strong ethanol peak	Fermentation appeared effective based on the chromatographic trend.

Table 4.5: Overall indicators of fermentation efficiency

The results presented in Table 4.5 show that fermentation efficiency was mainly supported by sugar reduction and ethanol formation. The presence of glycerol and minor organic acids further confirmed microbial activity during fermentation. These observations suggest that the HPLC-RID method was useful for evaluating fermentation behaviour in both grain-based and molasses-based samples.

4.10 Summary of Results

By finding sugars, oligosaccharides, ethanol, glycerol, and organic acids in the examined materials, the HPLC-RID study offered valuable insights into the fermentation process. While the standard solution aided in defending chemicals based on retention behaviour, the blank run verified that the chromatographic apparatus was appropproper Foris. Understanding substrate consumption and metabolite synthesis was aided by the chromatograms of the samples, which revealed the existence of chemicals relevant to fermentation.

The findings showed that fermented samples had significantly fewer sugar-related reactions. This implied that microbial metabolism involved the consumption of fermentable carbohydrates. Since sugars are the primary substrates used by microbes for energy synthesis and metabolite creation, the decrease of sugar peaks is a crucial indicator of fermentation success.

Ethanol was seen as a major fermentation product. Its strong response confirmed that alcoholic fermentation occurred successfully. The presence of ethanol along with a lower sugar response showed the conversion of available sugars into alcohol. This pattern is one of the strongest indicators of effective fermentation.

Glycerol was also detected in the samples. Its presence supported active yeast metabolism during fermentation. Although glycerol was not the major product, it provided added evidence of microbial activity. Organic acids such as lactic acid and acetic acid were detected at lower levels, showing minor side metabolic reactions.

The chromatographic profile was found to be influenced by the type of substrate when samples based on molasses and grain were compared. Because starch-derived sugars are involved, grain-based samples displayed a more intricate carbohydrate-related pattern. Samples made using molasses had characteristics of easily accessible soluble sugars.

This variation proved that the distribution of metabolites and fermentation performance are influenced by the content of raw materials.

For qualitative and trend-based fermentation monitoring, the analytical approach proved respectable dependability. Sample clarity was enhanced, and increased chromatographic performance was supported by proper sample preparation, which included dilution, centrifugation, and filtration. The interpretation was more trustworthy because blank, standard, and sample analyses were used.

Overall, the study showed that HPLC-RID may be used to track the development of fermentation. It made it easier to see how sugar was used, how ethanol was produced, and how secondary metabolites were formed. Based on chromatographic observations, the results confirmed that the fermentation process was active and efficient

Result area	Major finding
Blank run	No major interference was seen.
Standard run	Major compounds were found based on retention behaviour.
Sample profile	Sugars, ethanol, glycerol, and organic acids were detected.
Sugar use	Sugar-related responses were reduced in fermented samples.
Ethanol production	Ethanol response was strong and clearly saw.
Secondary metabolites	Glycerol, lactic acid, and acetic acid were detected.
Substrate comparison	Grain-based and molasses-based samples showed different chromatographic behaviours.
Method suitability	HPLC-RID was suitable for qualitative and trend-based fermentation monitoring.

Table 4.6: Summary of major findings of HPLC-RID analysis

The overall findings confirmed that HPLC-RID can be used as an effective analytical technique for fermentation monitoring. The method provided useful information about substrate use, ethanol formation, and secondary metabolite production in grain-based and molasses-based fermentation samples.

CHAPTER 5: CONCLUSION

The present study demonstrated the successful application of High-Performance Liquid Chromatography coupled with Refractive Index Detection (HPLC-RID) for monitoring fermentation behaviour in grain-based and molasses-based samples. The analytical method provided effective separation and identification of important fermentation metabolites including sugars, ethanol, glycerol, lactic acid, and acetic acid.

The chromatographic analysis confirmed progressive changes in metabolite composition during fermentation. A gradual reduction in sugar-related peaks along with an increase in ethanol peak intensity indicated active yeast metabolism and efficient substrate utilization. Differences in chromatographic profiles between grain and molasses samples reflected the variation in substrate composition and fermentation behaviour.

The use of optimized chromatographic conditions, proper sample preparation, filtration, and calibration procedures contributed to stable baseline response and reliable peak separation throughout the analysis. The study also highlighted the usefulness of HPLC-RID as a practical analytical tool for real-time fermentation monitoring in industrial and research applications.

Overall, the findings demonstrated that HPLC-RID is a reliable, reproducible, and effective technique for studying fermentation kinetics and monitoring biochemical changes during yeast fermentation processes.

FUTURE PERSPECTIVES

Although the present study successfully demonstrated the application of HPLC-RID for fermentation monitoring, further improvements can enhance analytical precision and industrial applicability. Future studies may include automated sampling systems for continuous real-time monitoring of fermentation processes under industrial conditions.

Advanced chromatographic techniques such as LC-MS, GC-MS, or HPLC coupled with UV/PDA detectors may be used for more detailed characterization of fermentation metabolites and trace compounds. Quantitative analysis using larger calibration datasets and statistical modelling can further improve analytical accuracy and process interpretation.

Future researchers may also investigate the influence of different yeast strains, fermentation temperatures, pH conditions, and nutrient compositions on metabolite production and fermentation efficiency. Comparative studies involving multiple industrial substrates could provide deeper understanding of fermentation kinetics and substrate utilization patterns.

The integration of modern analytical tools with industrial fermentation systems may contribute to improved process optimization, product

consistency, and large-scale bioethanol and biochemical production in the future.

Future work may also focus on:

- monitoring fermentation kinetics at different fermentation stages,
- comparative evaluation of different microbial strains,
- analysis of added industrial substrates,
- large-scale industrial fermentation monitoring and integration of chromatographic analysis with advanced analytical techniques such as LC-MS or GC-MS for detailed metabolite characterization.

The use of automated chromatographic systems and real-time fermentation monitoring approaches may further improve process efficiency and industrial fermentation control in future studies.

6. References

1. Dong, M. W. (2006). *Modern HPLC for practicing scientists*. Wiley-Interscience.

2. International Conference on Harmonisation (ICH). (2005). *Validation of analytical procedures: Text and method Q2(R1)*. ICH.

3. Nielsen, J., Villadsen, J., & Lidén, G. (2017). *Bioreaction engineering principles* (3rd ed.). Springer.

4. Stanbury, P. F., Whitaker, A., & Hall, S. J. (2017). *Principles of fermentation technology* (3rd ed.). Elsevier.

5. United States Pharmacopeia (USP). (2020). *General Chapter <621>: Chromatography*. United States Pharmacopeial Convention.

6. Walker, G. M. (2011). Pichia anomala: Cell physiology and biotechnology. *Antonie van Leeuwenhoek, 99*(1), 25–34.

7. Moldoveanu, S. C., & David, V. (2019). *Modern sample preparation for chromatography*. Elsevier.

8. Snyder, L. R., Kirkland, J. J., & Dolan, J. W. (2010). *Introduction to modern liquid chromatography* (3rd ed.). Wiley.